GROWTH FACTORS

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Preface

Growth factors, as the name implies, were originally discovered by virtue of their ability to promote cell multiplication in culture. The last few years has seen an explosion of discoveries in this area and growth factors are now implicated in many fields of modern biology including direct clinical applications as well as more fundamental scientific problems. This book is designed to provide an introduction to this increasingly significant class of intercellular signalling molecules.

The starting point of the text is the historical roots of growth factor research in the biology of mammalian cell proliferation. This leads into a more detailed consideration of the biochemistry of growth factors, their receptors and intracellular signalling pathways. This focus on the fundamentals is deliberate. The scope of growth factor biology is vast, increasing, and impossible to adequately cover in a simple monograph. It is therefore aimed to equip the interested reader with sufficient background knowledge to venture further into the specialized literature.

It is also difficult to write a book on growth factors without venturing into other fields. In particular, cancer biology, development biology, and neuroscience are beginning to have a profound impact on the understanding of growth factor function. This text must therefore be viewed as a status report. The definitive account is many years in the future.

I am very grateful to the many students, colleagues, and collaborators whose ideas and comments have been an essential stimulus to the composition of this book.

John K. Heath

Abbreviations

aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BSA	bovine serum albumin
CNTF	ciliary neurotrophic factor
DAG	diacylglycerol
EGF	epidermal growth factor
EGF-R	epidermal growth factor receptor
FGF	fibroblast growth factor
FSH	follicle stimulating hormone
G1	phase following the mitotic phase of the cell cycle
G2	phase following the S phase of the cell cycle
GAP	GTPase activating protein
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
HeLa	a carcinomatous cell type, named after Henrietta Lacks
IGF	insulin-like growth factor
IGF-BP	insulin-like growth factor binding protein
K-FGF/Hst	kaposi fibroblast growth factor
KGF	keratinocyte-derived growth factor
LAP	latency associated protein
LHRH	luteinizing-hormone releasing hormone
M-CSF	macrophage colony stimulating factor
MIS	mullerian inhibitory substance
M phase	mitotic phase of the cell cycle
MSV	moloney sarcoma virus
NGF	nerve growth factor
PDGF	platelet-derived growth factor
PIP2	phosphatidylinositol 4,5 bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PTH	parathyroid hormone
S phase	synthesis phase of the cell cycle following the G1 phase
TGF	transforming growth factor

TRE	TPA response element
TRH	thyrotrophin releasing hormone
VEGF	vascular endothelial cell growth factor
VVGF	Vaccinia virus growth factor

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1

An introduction to cell proliferation

1. Introduction

The ability to replicate and multiply is a fundamental and characteristic property of living organisms from the simplest single-celled bacterium to the most complex multicellular vertebrate such as ourselves. At the level of the individual cell, however, the biological demands are quite distinct. A multicellular organism is a community of cells whose multiplication must be matched to the overall requirements of the organism, and this demands that the behaviour of individuals cells must be subject to some form of coordinate control. In other words, the multiplication of cells within a multicellular organism is responsive to external signals generated by other cells.

In this chapter, the multiplication of mammalian cells is considered from a biological perspective. This definition of the behavioural characteristics of mammalian cell multiplication provides the basis for understanding the nature of the underlying mechanisms.

2. The cell cycle

The multiplication of any cell involves two essential phases—replication of the genetic material and equal partitioning of the replicated DNA into two daughter cells (mitosis). This process is quasi-repetitive in that, except in particular circumstances (described below), the processes are repeated in the daughter cells and in their daughter cells in turn. In addition, the two processes occur in a fixed order. The replication of DNA must proceed, and be completed, before it is partitioned into the cells of the next generation. There are some exceptions to this generalization. The most important, which occurs in all sexually reproducing organisms, is the generation of haploid gametes in which genetic information is distributed without prior replication (meiosis). In some specialized cells, DNA is replicated without a succeeding mitosis, leading to the production of individual cells with an increased DNA content. The processes involved in the

orderly completion of both phases in the life of a cell is accordingly called the cell cycle.

2.1 Measurement of the phases of the cell cycle

DNA replication and mitosis represent 'landmarks' in the cell cycle which can be readily observed and measured by the investigator. When cells undergo DNA replication they incorporate nucleotide precursors into their DNA and this can be detected by exposing the cells to chemically or radioactively labelled nucleotide precursors. As cells enter mitosis their chromosomes condense and become orientated on the mitotic spindle, which is followed shortly after by partitioning of the chromosomes and division of the cell into two daughters. The process of mitosis and its individual phases can be easily visualized under the microscope. In addition, when the cell divides, two daughter cells exist in place of the original parent. In the absence of cell death, a population of dividing cells increases in number. The practical significance of this is that the ability to observe and measure these landmark events provides the basis for defining the kinetic and regulatory properties of the cell cycle.

It is not possible to measure directly the duration of every phase in the cell cycle in a population of cells. Instead it is necessary to bring together a variety of approaches to build a composite picture of cell cycle characteristics of a population of cells undergoing multiplication. These approaches fall into three main categories, each of which provides certain types of information.

Firstly, it is possible to sample a population of cells at different times and determine experimentally either the total number of cells present or the fraction in S phase (the 'synthesis phase' of the cycle) or mitosis, or, by means of flow cytofluorimetry, determine the fraction of the population which have doubled their DNA content but have not yet divided. This sampling approach provides information on the overall properties of a population of cells, but little information on the duration of individual phases or the behaviour of individual cells within the population. Such a sampling approach can, however, become quite sophisticated when measurement of the two landmark events is combined, as, for example, in exposing cells to labelled nucleotide precursors for brief periods and then measuring the rate at which labelled cells reach mitosis.

The second approach involves the continuous observation of a population of dividing cells by time-lapse film recording. This permits detailed analysis of individual cell behaviour but is restricted to the measurement of mitosis or cell division, which are the only phases measurable by direct observation.

The third approach relies on the use of drugs which block progress through the cell cycle at either S phase (e.g. hydroxyurea) or mitosis (e.g. Colcemid). If such a drug-induced block to progress through the cell cycle is applied, as individual cells reach the point of the block further progress is arrested and a population of cells accumulates at a particular point in the cell cycle. This phenomenon can be used in two ways. Firstly it may be possible to measure the rate at which cells accumulate at the block, and thereby infer the rate of progress through the previous phases, or cells may be allowed to accumulate at the block

for a fixed period, after which the block is released by removing the drug, and the time taken to reach the next observable landmark event measured. This type of experiment is especially useful in measuring the duration of periods of the cell cycle in which no easily measurable landmark event is occurring. The drawback of this type of approach is, of course, the possibility that the duration of cell cycle events being measured are influenced by the action of the drug.

2.2 Other features of the cell cycle

The combined application of the methods described reveals that the 'typical' eukaryotic cell cycle contains 'extra phases' in which the cells are neither undergoing DNA synthesis nor actively dividing. These have been termed G1, which is the period after the daughter cells have been formed but before they enter DNA synthesis, and G2, which is the phase between the completion of DNA synthesis and the onset of mitosis. In the G1 phase, cells have typically a diploid DNA content (2C) and in the G2 phase, they have twice the diploid DNA content (4C). It is now possible to consider the cell cycle, from the 'birth of a cell', as therefore comprising four phases: G1, the period of DNA replication (or S phase), G2, and mitosis (*Figure 1.1*). Each of these phases can be either directly measured or inferred and it is therefore possible to create a composite kinetic analysis of cell behaviour in each of the four cell cycle phases.

2.3 The kinetic properties of the phases of the cell cycle

Combining a large body of information obtained from different types of cells by different experimental means, allows us to make some general statements about the duration of the individual cell cycle phases.

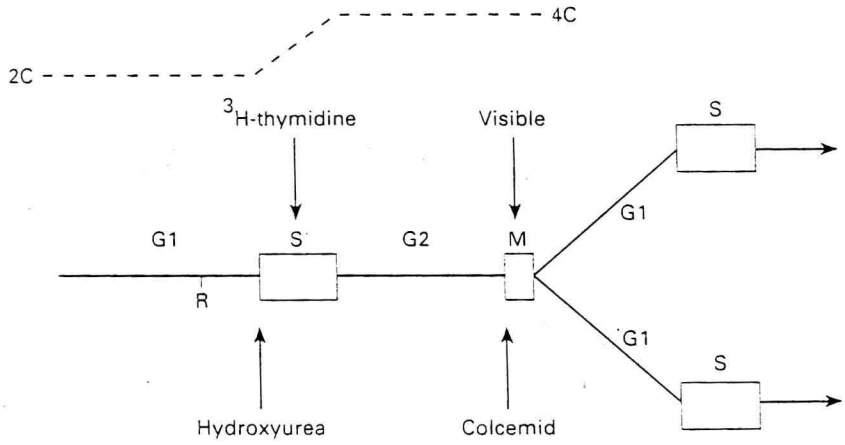

Figure 1.1. The phases of the mammalian cell cycle. S phase can be detected by incorporation of labelled nucleotide precursors and mitosis can be visualized under the microscope. Progress through phases can be specifically blocked by drugs such as hydroxyurea (S phase) or Colcemid (M phase). Progress through S phase entails a doubling of DNA content from 2C to 4C. R = restriction point.

Firstly, mitosis is a relatively rapid affair, often occupying less than an hour, and which exhibits little variation in duration between cells with widely different overall rates of multiplication.

Secondly, S phase typically takes a matter of a few hours to complete (although there are striking exceptions, such as in the early embryos of certain species in which S phase occurs in 15–30 min). Likewise, G2 occupies a few hours in the mammalian cell's cycle except in special cases such as the unfertilized egg, in which G2 is prolonged indefinitely until it is completed suddenly upon fertilization. The duration of both S phase and G2 may vary slightly between individual cells within a population but this variation is, in the majority of cases, not correlated with the overall distribution of cell cycle times of the entire population, and, in particular, S and G2 have roughly the same duration in populations of cells whose rates of multiplication are quite dissimilar.

It is the duration of the G1 phase which appears to be the major determinant of the duration of the cell cycle in eukaryotic cells. The influence of the G1 phase arises, not necessarily from its absolute duration, but rather from the fact that it is a period which exhibits considerable variation between populations of cells multiplying at different rates. It is, in fact, a noteworthy feature that individual eukaryotic cells within a population rarely exhibit identical cell cycle times. In most cases the overall duration of the cell cycle in an apparently identical population of cells is variable. Furthermore, the degree of variability increases as the overall rate of population doubling decreases, and this arises because as the rate of population doubling slows, the duration of G1 extends and at the same time becomes more variable within a population, whilst the other phases remain fairly constant in duration. In the extreme case the G1 phase of the cell cycle can be considered to be 'indefinitely extended' and further progress into S phase, G2, and mitosis is halted.

Examination of the distribution of either the overall cell cycle or the duration of G1 reveals that the variability is not simply 'random' or Gaussian in nature but instead appears to become skewed towards longer and longer cell cycle times as the overall cell cycle becomes extended (*Figure 1.2*). Within this 'skewed distribution' there are cells whose G1 phase duration is little different from those of a more rapidly dividing population and other cells whose G1 phase has become 'infinitely extended' and, in effect, never divide again. As population doubling times slow further, it is this latter category of cells whose representation within the population increases. This allows us to define an additional parameter of population cell cycle kinetic: the 'growth fraction' of a population of cells, which is the proportion of cells within a population which will give rise to daughter progeny within a measurable time frame.

The variability of cell cycle times in eukaryotic cells has exercised a considerable degree of fascination for mathematicians, who have attempted to fit population cell cycle data to different types of mathematical distribution, and thereby deduce features of the mechanism underlying the variation. This has proved to be a difficult (and so far futile) exercise. What does emerge most clearly from consideration of the eukaryotic cell cycle is, however, the fact that variability,

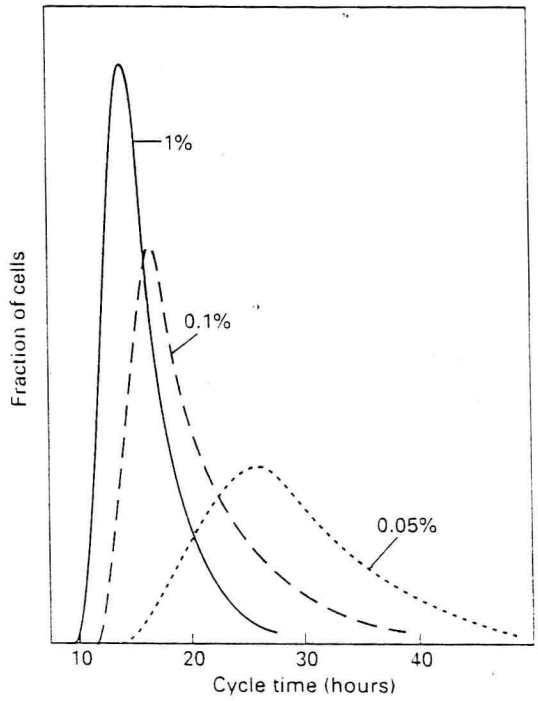

Figure 1.2 Distribution of cell-cycle times in populations of 3T3 cells grown in different concentrations of serum. As the overall rate of multiplication slows the distribution of cell-cycle times becomes more variable.

and hence control and regulatory processes, occurs predominantly in the G1 phase of the cell cycle. Any attempts at molecular explanations of cell cycle regulation and control must therefore focus, at least in part, on the biochemical processes of G1.

3. Senescence

So far the eukaryotic cell cycle has been considered to be an indefinitely repetitious, albeit variable, process. Common sense consideration of the human body, however, argues that this is an oversimplification. Whilst some cells in the adult, such as those of the skin or the blood, proliferate throughout most of our adult lives, others, such as those of the nervous system, cease dividing soon after birth and never divide again in the lifetime of the organism. Closer consideration of tissues such as the skin or the blood suggests, in fact that even within an actively dividing tissue, cells cease to divide and eventually die or are removed, and the two processes are normally in balance.

The eventual loss of the ability to divide is in fact a fundamental property of most types of cells within the body. The process by which cells lose the ability to produce progeny cells is termed 'senescence'. In a senescent population of cells the growth fraction has declined to zero and no further increase in population is possible. As just described, even within a population with cells actively undergoing cell proliferation, there may be a fraction of senescent cells that never divide.

The phenomenon of senescence can most easily be studied by longitudinal observation of a population of cells grown outside the normal habitat of the body, in culture. If normal cells, for example from the skin, are removed and grown in culture, their behaviour can be studied over many generations under relatively controlled conditions. In most cases it is found that normal cells divide and multiply quite rapidly, but as time and the number of generations increase, the overall rate of population increase begins to slow and eventually reaches a plateau (*Figure 1.3*). At this point the growth fraction has dropped to zero and all the cells remain indefinitely in G1. Closer inspection of this phenomenon shows that senescence does not occur instantaneously after the same period of chronological time or number of cell divisions but rather it is a progressive process. As a population of cells approaches senescence, cell cycle times increase and become progressively more heterogeneous and, at the same time the growth fraction progressively decreases (1). In other words the process of senescence, whilst seemingly an obligatory fate of all cells, exhibits considerable cell-to-cell variation.

The phenomenon of senescence also exhibits some unexpected properties when different types of cells are considered. Firstly, extensive studies of human fibroblasts derived from skin biopsies from different individuals show that the average number of cell doubling that occurs before the plateau phase is relatively constant from individual to individual, being, in this instance, approximately 50 generations (2). Secondly, the number of generations required for a population to reach the plateau phase is strongly influenced not by the species of origin, but by the exact type of cell in question. Whilst skin fibroblasts reach a plateau after approximately 50 generations, some cell types from the early embryo can plateau and senesce after only six to seven generations in culture. This means that cellular senescence appears to be a 'programmed' phenomenon whose kinetics are ultimately controlled by the identity of the type of cell.

4. Quiescence

Senescence is, as described above, an essentially irreversible phenomenon. A population of cells may, however, cease to proliferate, but retain the ability to divide further. This phenomenon is termed 'quiescence'. The state of quiescence is most usually brought about by manipulation of the environment of the cell, and how this occurs is described in detail in Section 7. The important point to note, however, is that quiescence is a reversible phenomenon. If cells are rendered

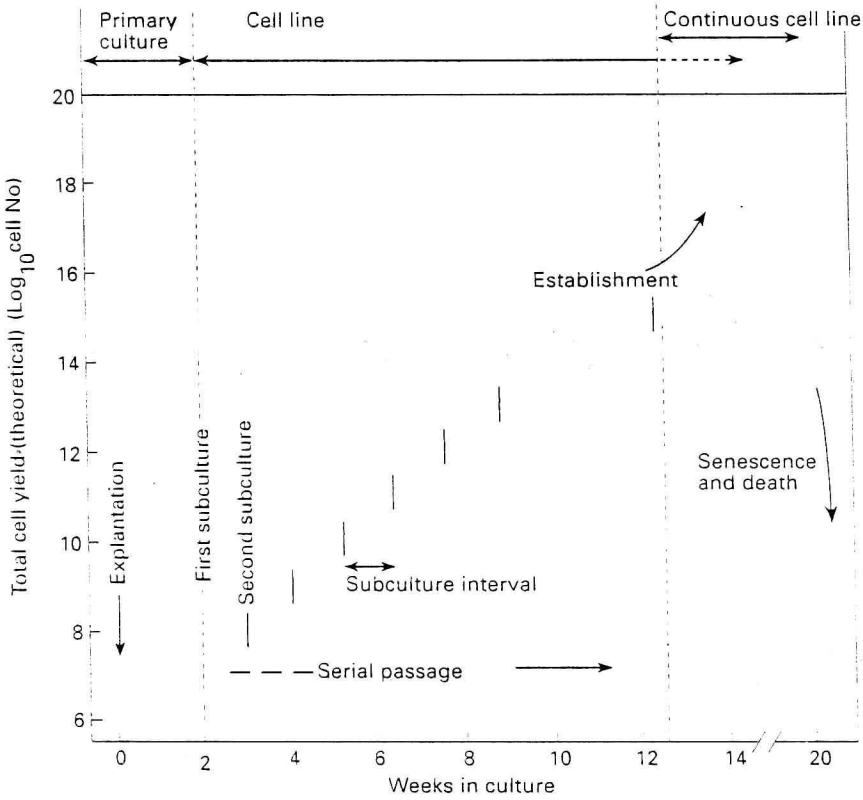

Figure 1.3 Evolution of a cell line. The vertical axis represents total cell growth (assuming no reduction at passage) on a log scale; the horizontal axis represents time in culture on a linear scale, for a hypothetical cell culture. Although a continuous cell line is depicted as arising at 12½ weeks it could, with different cultures, arise any time. Likewise, senescence may arise at any time, but for human diploid fibroblasts it is most likely between 30 and 50 cell doublings or 10–20 weeks depending on the doubling time. (Reproduced from ref. 2; with permission.)

quiescent by alteration or manipulation of their environment, they can be induced to re-enter the cell cycle and proliferate by restoration of the original conditions. Closer inspection of cells whose multiplication has been brought to a halt by quiescence reveals that they are halted, not randomly, but specifically in the G1 phase of the cell cycle, and the process of departure from quiescence and reinitiation of cell multiplication must necessarily involve events occurring in G1.

Senescence and quiescence can be considered as being analogous phenomena; they both involve cessation of multiplication and progress through the cell cycle. Whilst senescence is 'programmed' and essentially irreversible, quiescence is

dependent upon environmental stimuli. In both cases, however, the process is initiated by failure to progress through G1. Taken together with G1 as the major variable phase in the cell cycle, this leads to the conclusion that G1 is a phase in which prominent control points (external or internal) in the cell cycle are exerted.

5. Establishment of cell lines

Whilst the majority of normal cells eventually undergo senescence there are some important exceptions. The most significant in the present context is the phenomenon of 'establishment'. Establishment occurs in sub-populations of cells within a senescent population and becomes manifest when the population cell number reaches a plateau but then begins a second phase of increase (*Figure 1.3*). These 'established cell lines' which have 'escaped' senescence are now to all intents and purposes, immortal and never undergo senescence (3).

The propensity to yield established cells appears to vary from species to species and rodents, such as the mouse or rat, appear especially prone to producing established cell lines, whereas establishment seems extremely rare in the case of human cells. In all cases, however, established cells appear to have undergone chromosomal rearrangements and duplications and it appears very probable that the process of establishment has some form of genetic basis. The major practical issue is, however, that established cell lines represent a permanent and uniform population of cells whose properties do not change over time. The second key issue is the fact that established cells, whilst unable to senesce, do still exhibit quiescence when subject to appropriate environmental manipulations and therefore established cell lines (such as mouse 3T3), despite their abnormalities, have become the most common cell type for biochemical and biological investigations into the control mechanisms of the cell cycle (3, 4).

6. Cell proliferation and the environment

It has already been mentioned that the multiplication of cells can, as manifested in the phenomenon of quiescence, be controlled by external environmental influences which appear to act on control points in the G1 phase of the cell cycle. The ability to control cell multiplication through manipulation of the environment is not only a powerful practical tool but it also provides a crucial entry point into the biochemical analysis of the control of mammalian cell proliferation. The philosophy behind this approach is that determination of the identity of the critical environmental determinants of cell proliferation, in particular those responsible for the phenomenon of quiescence, could lead to analysis of their action at the molecular level and thence into biochemical dissection of the intracellular control mechanisms themselves. The development of this approach occupies the remaining chapters of this book, but at this point it is valuable to consider further

the biological nature of the response of the eukaryotic cell cycle to environmental factors.

Considerable effort has been expended in the past hundred years in trying, largely by empirical means, to find appropriate conditions to permit the propagation of mammalian cells in culture. For, whereas bacteria and fungi will, in most cases, proliferate in the presence of simple nutrient sources, the requirements of vertebrate (principally mammalian) cells are significantly more complex. In particular, mammalian cells appear to require environmental factors over and above simple chemical or macromolecular nutrients.

The identification of these extra requirements has been a lengthy and difficult process and, for many types of cells, is by no means complete. The experimental methods involved in resolving these issues comprise two contrasting approaches. The first and historically most common involves the attempt to recreate, as faithfully as possible, what is thought to represent the 'natural' environment of cells within the body. Having found conditions which permit cell proliferation, the next step is to begin biochemical fractionation with the objective of identifying the active components. The second, and more recent method, is to begin with a simple mixture of nutrients and to 'add back' ingredients until an optimal mixture for cell proliferation is obtained. As will be seen below these two approaches converge on the identification of a core set of molecules required for the survival and, most importantly proliferation of mammalian cells in culture.

7. The role of serum

The earliest successful attempts at growing mammalian cells in culture involved the use of clotted blood, either in the form of solid plasma clots (such as would occur at the site of wound injury) or serum (the soluble fraction of clotted blood). It was noted in these cases that serum (either solid, in the form of the clot, or liquid) was essential if cells were to proliferate, whereas viability could be maintained in the presence of simple buffered salt solutions fortified with nutrients. By a converging process of optimizing both the nutrient mixture components and the sources of serum, it proved possible, by the early 1950s, to grow a limited number of cell types in culture quite readily for prolonged periods of time (up to and including the plateau phase) under reproducible conditions. This technical achievement had two significant implications. It became possible to define experimentally in what way the presence of serum influenced progress through the cell cycle and thence to attempt to define the biochemical components of serum responsible.

The most significant investigations into the role of serum in the control of mammalian cell multiplication centred on the analysis of the established mouse cell line 3T3. Robert Holley and co-workers (5) noted that if 3T3 cells were placed in culture in a nutrient mixture supplemented with serum, they multiplied, then the population plateaued and entered quiescence (*Figure 1.4(a)*). The number of divisions that occurred before quiescence was related to the amount